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ORIGINAL PAPER

Genetically engineered Thompson Seedless grapevine plants designed for fungal tolerance: selection and characterization of the best performing individuals in a field trial

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Abstract The fungi *Botrytis cinerea* and *Erysiphe necator* are responsible for gray mold and powdery mildew diseases, respectively, which are among the most devastating diseases of grapes. Two endochitinase (*ech*42 and *ech*33) genes and one *N*-acetyl-β-D-hexosaminidase (*nag*70) gene from biocontrol agents related to *Trichoderma* spp. were used to develop a set of 103 genetically modified (GM) 'Thompson Seedless' lines (568 plants) that were established in open field in 2004 and evaluated for fungal tolerance starting in 2006. Statistical analyses were carried out considering transgene, explant origin, and plant response to both fungi in the field and in detached leaf assays. The results allowed for the selection of the 19 consistently most tolerant lines through two consecutive years (2007–2008 and 2008–2009 seasons). Plants from these lines were grafted onto the rootstock Harmony and established in the field in 2009 for further characterization. Transgene status was shown in most of these lines by Southern blot, real-time PCR, ELISA, and immunostrips; the most tolerant candidates expressed the *ech*42–*nag*70 double gene construct and the *ech*33 gene from a local *Hypocrea virens* isolate. *B. cinerea* growth assays in Petri dishes supplemented with berry juices extracted from the most tolerant individuals of the selected population was inhibited. These results demonstrate that improved fungal tolerance can be attributed to transgene expression and support the iterative molecular and physiological phenotyping in order to define selected individuals from a population of GM grapevines.

Keywords Botrytis tolerance · Transgenic grapevine · Field trial · Chitinase · 'Thompson Seedless'

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Introduction

Grapevine growers invest millions of dollars to mitigate the impact of fungal diseases (Haggag 2008). Among these diseases, gray mold caused by the necrotrophic fungus *Botrytis cinerea* Pers. [tel. *Botryotinia fuckeliana* (de Bary)] attacks leaves or inflorescences and results in serious economic damage (Haggag 2008). Flowers typically become infected during the late blooming period without showing symptoms. Then, the pathogen enters into the young fruit at a very early stage of development (Elad et al. 2004; Keller et al. 2003). Low temperatures and other environmental conditions cause gray mold onset; disease results in significant pre- and post-harvest losses. On the other hand, infection caused by the biotrophic fungus *Erysiphe necator*; sin. *Uncinula necator* Burr., the causal agent of powdery mildew, also depends on the timing of the first infection. Infections can occur in all succulent tissues on a grapevine (including the stem, leaves, and fruits), decreasing the rate of photosynthesis and leading to reduced berry sugar content in table grapes and unpleasant flavors in wine. Infections that occurred at early developmental stages of the fruit, i.e., a few days after fruit setting, will cause berry stunting and scarring.

Although resistance sources to many fungal diseases have been found in *Vitis* species and their transfer to *Vitis vinifera* cultivars has been carried out with some success (Peterlunger et al. 2003; Figueiredo et al. 2008; Ramming et al. 2011), resistant varieties of commercial value derived from these interspecific hybrids are not yet available. Thus, the use of alternative procedures such as gene transfer have garnered interest due to the possibility of establishing disease tolerance or resistance in elite grapevine varieties and rootstocks without changing other genotype-specific characteristics.

Several approaches have been used in plants to engineer fungal resistance (Wally and Punja 2010). One approach includes the use of anti-fungal enzymes, e.g., chitinases or glucanases, from different sources, including plants (Chye et al. 2005; Vellicce et al. 2006) or mycoparasitic fungi (Lorito et al. 1998; Bolar et al. 2001; Emani et al. 2003; Liu et al. 2004). A 42 kDa extracellular endochitinase (*ech*42, EC 3.2.14) from *Trichoderma harzianum* and *Trichicoderma virens* has been described as playing a key role in the biological control of *B. cinerea* and other pathogens (Schuster and Schmoll 2010) and the use of the corresponding genes has led to the generation of tolerant transgenic lines in several species such as potatoes and tobacco (Lorito et al. 1998), apples (Bolar et al. 2001), petunias (Esposito et al. 2000), broccoli and canola (Mora-Avilés and Earle 2004), cotton (Emani et al. 2003), rice (Liu et al. 2004), and grapes (Kikkert et al. 2000). Another endochitinase gene identified in *Trichoderma*, *ech*33, whose protein showed significant similarity to some pathogenic response-associated class-III plant chitinases (Limón et al. 1995), was used for tolerance in tobacco to biotic (*Rhizoctonia solani* and *Pseudomonas syringae* pv *tabaci*) and abiotic stresses, such as heavy metals (Dana et al. 2006). In addition, a *T. harzianum* P1 *N*-acetyl-β-D-hexosaminidase (*nag*70, EC 3.2.1.52) has also been used to generate transgenic 'Marshall McIntosh' apples and produce lines with enhanced tolerance against *Venturia inaequalis* (Bolar et al. 2001). Higher tolerance levels have been obtained by stacking several chitinolytic genes from *Trichoderma* compared with using single genes (Dana et al. 2006; Liu et al. 2004; Bolar et al. 2001).

Since 2002, a high-throughput *Agrobacterium tumefaciens* (updated scientific name: *Rhizobium radiobacter*)-mediated gene transfer of somatic embryos of *V. vinifera* 'Thompson Seedless' (TS) has been used at La Platina Research Station of the National Agriculture Institute (Santiago de Chile) (Reyes et al. 2005; Tapia et al. 2009). Transgenic lines were developed using *ech*42 and *nag*70 from *T. harzianum* P1 and *ech*33 from a local isolate of the teleomorph of *T. virens*, *Hypocrea virens* (Acc. no. FJ358733). Of the 3,000 transgenic lines that were established in the greenhouse (Hinrichsen et al. 2005), 103 lines, from which 568 plants were produced, were released in the field in September 2004. This study reports the field evaluation of transgenic TS during two consecutive seasons and the identification of transgenic lines exhibiting tolerance to *B. cinerea* and *E. necator*. Controlled leaf infections and symptoms at the field level due to natural disease occurrence were used. The statistical analysis and further molecular characterization used to define a tolerant population are described.

Materials and methods

Plant materials

Two different TS plant materials were used: certified TS plants commercially available from Brown Group, Inc., (Los Andes, Chile) and a TS accession (GI) introduced from the United States (Dennis Gray, Apopka Station, University of Florida, USA). Plantlets from these accessions were grown in C2D medium (Chée and Pool 1987) supplemented with 4 μM 6-benzyladenine to induce multiple budding in a period of about 30 days. Cultivation chambers for these materials were set at 24 ± 2 °C with a constant photoperiod of 16 h light/8 h darkness.

Somatic embryogenesis

Apical and axial buds with 2–4 leaves were cut from in vitro-grown TS and GI plants. Buds were excised using a stereoscopic lens and incubated for callus induction in NB2 medium following the general procedures introduced by Li et al. (2001) by cultivation in darkness for 30 days at 24 ± 2 °C to form pro-embryogenic calli. Pro-embryogenic masses were transferred to X6 medium (Li et al. 2001) and kept at the same temperature under a 16 h light/8 h darkness photoperiod for up to 90 days. Somatic embryos were harvested from the masses according to their developmental stage and maintained on the same medium. To induce development, embryos at cotyledonary stage were transferred to C2D medium supplemented with 0.4 μM BA, 0.25 μM indolbutyric acid (IBA), 2 g/L glycine, and 0.5 g/L activated charcoal and kept under the same conditions of temperature and photoperiod for 20 days. The total time involved in the entire induction and development of embryos was at least 110 days. Somatic embryos were used for the gene transfer experiments.

Gene constructs

Constructs for ech42 and nag70 genes

Constructs for the *ech*42 and *nag* genes from *T. harzianum* P1 were developed at Cornell University (New York, USA), as previously described by Bolar et al. (2001).

Gene construct for ech33

We built the construct pCAMBIA2300-34S-ech33 by cloning the *ech*33 gene from a local *H. virens* isolate (GenBank accession number FJ358733). The *ech*33 gene was obtained by polymerase chain reaction (PCR) from genomic DNA prepared as described by Müller et al. (1998). Primers chi33for 5′-ATGCCT TCCTTGACTGTTCTTGCG-3′ and chi33rev 5′-TTA CCTCAAAGCATTGACAACCTG-3′ were designed with Primer3 software v. 0.4.0 (Rozen and Skaletsky 2000; http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) using three previously described sequences as a reference (De la Cruz et al. 1992; Hayes et al. 1994; Limón et al. 1995). A gene fragment of 1,157 base pairs was cloned into pGEM-T (Promega, Madison, WI, USA) and sequenced (Macrogen, Seoul, South Korea) (EMBL EBI sequence accession number FJ358733.1). This construct was used as a template for a new round of PCR, in which the same primers were tailed with *Spe*I sites in order to generate a *Spe*I-*ech33*-*Spe*I fragment that was cloned into the same site in pUC18FMV (Marlene Rosales, Pontificia Universidad Católica de Chile). A 34S *Figwort mosaic virus* (FMV) promoter—*ech33*-35S CaMV terminator fragment was excised from the pUC18FMV-ech33 construct by restriction with *Pst*I and cloned into the same site in pCAMBIA2300 (CAMBIA, Australia) to generate the final construct pCAMBIA2300-34S-ech33.

Gene transfer

In each gene transfer experiment, 200 mg of embryogenic mass was pre-conditioned in DM medium (Driver and Kuniyuki 1984) for 14 days in darkness. Embryo cells were incubated for 20 min in a solution ($OD_{600} = 0.6$) of *A. tumefaciens* EHA105-harboring vectors expressing the *ech*42, *ech*33, or (*ech*42 + *nag*70) genes and co-cultured for 2 days in darkness. Infected grape cells were washed three times with distilled water and cultured in Petri dishes containing DMcck medium (DM supplemented with 200 mg/L carbenicillin, 200 mL/L cefotaxime, and 100 mg/L kanamycin) for 21 days using the same photoperiod and transferred into modified X6cck medium (X6 supplemented with 200 mg/L carbenicillin, 200 mL/L cefotaxime, and 200 mg/L kanamycin) in Petri dishes for 60 days under the same photoperiod. To complete

development, embryos at the cotyledonary stage were transferred to Petri dishes containing C2D medium supplemented with 0.4 μM BA, 0.25 μM IBA, 2 g/L glycine, and 0.5 g/L activated charcoal for 20 days. Plantlets were selected and cultured in C2D medium supplemented with 5 μM BA, 0.5 μM IBA, and 1 mg/mL glycine for acclimatization. All these procedures were carried out at 24 ± 2 °C.

DNA extraction and characterization of putatively transgenic plantlets by PCR

Total DNA isolates from regenerated plantlets were obtained by processing approximately 100 mg of leaf tissue from shoots of rooted regenerants using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) following the protocol described by the manufacturer. Preliminary PCR analyses were set using these isolates using primers complimentary to the neomycin phosphotransferase II (*npt*II) gene (Urtubia et al. 2008) and the *V. vinifera stilbene synthase* gene as internal control (primers STS_F 5′-TTGACCGAA GAAATGCTTGAGGAG-3′ and STS_R 5′-AAAAG GGCTTGGCCAACTAAAGAG-3′) for the amplification of a 430 bp fragment.

Establishment of transgenic lines in open field

Two vineyard experiments were established for the present work. The first experiment was established with all transgenic plants that were positive according to *npt*II PCR analysis. For acclimatization, plants were transferred to a greenhouse using 8 × 20 cm plastic bags containing sterile soil (200 mL). Cultivation conditions in the greenhouse were 24 ± 2 °C with constant photoperiod of 16 h light/8 h darkness. After 5–10 months, plants were pre-conditioned in a shadehouse for 2 weeks before planting in open field in a mother block (MB). Planting distances used in MB were 50 cm between plants and 2.5 m between rows, in a completely random distribution of 4–6 replicates per line. Each plant in the field was labeled using a barcode system reporting data on source, vector, line, and plant (Hinrichsen et al. 2005). A total of 568 genetically modified (GM) plants (103 GM lines; 81 TS lines generating a population of 461 and 22 GI lines generating a population of 107) and 60 non-transgenic TS control plants were successfully established. The TS control population was obtained from somatic embryos without gene transfer. A second experiment was carried out after the last season of evaluations for plants in the MB; five dormant canes were selected from each of the 20 tolerant top-ranked lines and from one TS control, grafted onto Harmony rootstocks and re-planted in another section of the field. Planting distances were 2.5 m between and across rows to mimic commercial production conditions. Grafted plants were randomly distributed. Nineteen GM lines were successfully established in the second experiment.

Evaluation of tolerance to *B. cinerea* infection

An isolate of *B. cinerea* obtained from naturally infected grape berries (Muñoz et al. 2002; Rigotti et al. 2002) was prepared and maintained by plating on potato-dextrose-agar (PDA) in Petri dishes at 5 °C in the dark. Inoculation dishes were prepared, allowing for complete fungal growth at 20 °C under a photoperiod of 16 h of light and 8 h of darkness for 6–7 days. Leaves were collected from plant shoots between the fourth and sixth node and inoculated in the laboratory by contact on their adaxial surface with agar slices extracted from prepared fungal dishes using a puncher (7 mm diameter) and incubated at 18–20 °C using a photoperiod of 16 h light/8 h darkness. Control infections were carried out by incubating fungal agar slices on adaxial surfaces of 60 leaves from 6 to 8 TS plants from the same field and evaluated by time course analyses in order to define the best inoculation time. Data acquisition was set according to the infection pattern in these control leaves in which spots reached 2 cm in diameter (usually 72 h post-infection). Infections on GM individuals were measured as necrotic spots (in pixels) by image scanning of challenged leaves. Scoring times for these assays were between 4 and 6 weeks after flowering in 2007–2008 and 2008–2009.

Powdery mildew disease ranking

Disease annotations of *E. necator* infections were made in the transgenic and control individuals planted in the field using a modified scale for descriptors for grapevines (Descriptors for Grapevine; IPGRI, UPOV, OIV (1997); using scale values of 1, 3, 5, 7, and 9). Infection degrees were scored as follows: 1—very low (tiny spots or no symptoms, neither visible sporulation nor mycelium); 3—low, limited patches smaller than 2 cm in diameter, limited sporulation and

mycelium; 5—medium (patches usually limited to a diameter of 2–5 cm); 7—high (vast patches, some limited, strong sporulation, and abundant mycelium); 9—very high (vast unlimited patches or totally attacked leaf blades, strong sporulation, and abundant mycelium). All of the leaves of each GM and control plants (6–8 TS individuals) were assayed twice per season and scored for the indicated symptoms. Scoring times for young leaves were 3 weeks after flowering and mature leaves after harvest, in 2007–2008 and 2008–2009 seasons.

Statistical analyses

Data values obtained from infection pixels (*B. cinerea*) or disease annotations (*E. necator*) were ranked and processed by Kruskal–Wallis test in order to evaluate the null hypothesis that the medians within each of the columns is the same. The data from all the columns was first combined and ranked from smallest to largest. The average rank was then computed for the data in each column. Median notch in the columns were compared and represented using box-and-whisker plots. Comparisons considered explant source and transgene used as variables. The average rank for these values was computed to identify medians that were significantly different from each other. Statistically significant differences amongst the medians at the 95.0 % confidence level were defined when the P value was <0.05. All the statistical tools were executed using Statgraphics Centurion XV software (Statpoint Technologies, Warrenton, VA, USA).

Characterization of plants tolerant to *B. cinerea* and *E. necator* infections

Lines located in the first quartiles and down in the lower whiskers from both infection data sets were filtered using MS-Excel datasheets (Microsoft, Redmond, WA, USA) and the 20 top-ranked lines were selected for the second experiment. The successfully established lines were further characterized.

Characterization of the most tolerant plants

Real-time PCR exogene quantifications

Design of calibrator plasmids Three calibratorplasmids were built for each target gene (*ech*42, *ech*33, and *nag*70) by cloning into the calibrator plasmid p-*nptII*/*chi* (Dalla Costa et al. 2009). For cloning, target genes were amplified by PCR using *Apa*I- and *Sac*I-primer adapters (in bold), as follows: *ech33*_for: 5′-TTA **TCC GCG G**TT GAA CGG GGC CGA ACA T-3′, *ech33*_rv: 5′-AAT **GGG CCC** CTC GGC GGC GAT TCC TA-3′, *ech42*_for: 5′-ATA T**GG GCC C**TC GGC GTA GGC ATC TCC A-3′, *ech42*_rev: 5′-ATA T**CC GCG G**GG TCG CGT CGG ACA TCA C-3′, *nag*_for: 5′-TTT **GGG CCC** CCC AGC CAC GAT TGA ACG-3′, and *nag*_rev: 5′-AAA **CCG CGG** CGC CCA GGG TCT TTC ACC-3′. Amplifications were performed on a PCR Thermocycler Tgradient (Biometra, Göttingen, Germany) using a first denaturing step of 9 min at 95 °C, followed by 45 cycles of denaturation, annealing, and extension of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, respectively. A final extension at 72 °C for 5 min was used. Primers were designed with Primer3 software. Amplicons (*Apa* adapter-target gene-*Sac* adapter) and calibrator plasmid (p-*nptII*/*chi*) were double digested with *Apa*I and *Sac*I (Promega, Madison, WI, USA), purified from 1 % agarose gels using the NucleoSpin Extract II kit (Machinery-Nagel, Düren, Germany). Ligation reactions were carried out mixing linearized p-*nptII*/*chi* and the corresponding *ech42*, *ech33*, or *nag* digested amplicons in a 3:1 insert:vector molar ratio during an overnight incubation at 4 °C in the presence of T4 DNA ligase (Promega). Ligation mixtures were used in *E. coli* JM109 (Promega) transformation and positive clones were used for plasmid purification using the QIAprep Spin Miniprep Kit (Qiagen). Target gene calibrator plasmids were checked by sequencing in an ABI 3730xl using pUC/M13 forward and reverse universal primers. Plasmid solutions were prepared in water at 10^8 copies/μL, subdivided into 100 μL aliquots, and stored at −20 °C.

Real-time PCR amplifications The reactions were performed in 96-well reaction plates on the iCycler iQ Thermocycler (Biorad) in a 25 μL final volume. The reaction mix contained 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 100 ng of genomic DNA, 0.3 μM primers, and a 0.2 μM specific Taqman probe. Primers and Taqman probes for *chi* and *nptII* amplifications were as in Dalla Costa et al. (2009), while the primers for *ech33*, *ech42,* and *nag*70 were the same as used for the design of calibrator plasmids without adapter sequences. Taqman probes

were: *ech33*_RT_probe: FAM-5′-CAGCAGCCGAT TGAGCAGCACCT 3′-TAMRA, *ech42*_RT_probe: FAM-5′-TGAACTTCCAAGCAGACGGCACTGT-3′-TAMRA, and *nag*_RT_probe: FAM-5′-TGTCGCTG CCAAGAGTCAGGTTCCA-3′-TAMRA. The thermal profile was: uracil DNA glycosylase PCR decontamination for 2 min at 50 °C and 2 min at 95 °C, followed by 50 cycles of denaturation and annealing/extension for 15 s at 95 °C and of 1 min at 60 °C, respectively. Standard curves for *chi*, *nptII,* and exogenes were built by means of four decreasing concentrations of the respective multiple-target plasmid calibrator, in a serial dilution of 1:5 (1,000,000, 200,000, 40,000, and 8,000 plasmid molecules). The number of inserted exogenes in transgenic plants was calculated with the following formula: (exogene CN/*chi* CN) × 2. In the formula, the copy number (CN) of the *chi* endogene and the specific exogene are the mean values of two replicate threshold cycles (Ct) obtained with the iCycler iQ optical System Software, version 3.0a (Biorad). For each plant sample, two real-time PCR (qPCR) reactions were carried out and the final exogene CN value is the mean of the two measures. Nuclease-free water was used as a negative control.

Southern blot hybridizations

DNA extraction from the selected plants was conducted according to the procedure of Steenkamp et al. (1994). Fifteen µg of *Hind* III-digested genomic DNAs (New England Biolabs, Ipswich, MA, USA) was electrophoretically resolved on 1 % (w/v) agarose gels and transferred to positively charged nylon membranes (Roche Diagnostics Corporation, Indianapolis, IN, USA) after 48 h using 20× saline-sodium citrate (SSC) following the manufacturer's instructions. Hybridization on membranes was carried out for 3 h using the pre-hybridization buffer [50 % formamide deionized; 5× SSC; 0.1 % *N*-lauroylsarcosine; 0.02 % sodium dodecyl sulfate (SDS); 2 % blocking reagent (Roche, Basel, Switzerland)] at 37 °C and 16 h of hybridization at 42 °C using 5× SSC in the presence of the probe. Two low-stringency washes (2× SSC + 0.1 % SDS) were performed for 5 min at 37 °C and were followed by two high-stringency washes (0.5× SSC + 0.1 % SDS) for 15 min at 60 °C. We prepared a dioxigenine 11-dUTP alkali-labile (Roche) *npt*II probe following the manufacturer's instructions using primers NPTIIF-5′-ATGAT TGAACAAGATGGATTGCACG-3′ and NPTIIR-5′-CGAATGGGCAGGTAGCCGGATCAAGC-3′, which generated a 400 bp fragment. Hybridizations were detected using alkaline phosphatase-conjugated anti-DIG antibodies and revealed using the CDP-Star (Roche) reactives and Amersham Hyperfilm ECL films (GE Healthcare, UK), following the manufacturer's instructions. The size marker (V2) was built by [*ech*42-*nag*70] vector (Faize et al. 2003)/*Hind*III digestion. Electrophoretical separation and blotting for V2 followed the same experimental procedures as described. Detection of V2 was carried out by hybridization with a 35S probe prepared using the same experimental procedures as for *npt*II. The 35S probe was prepared by PCR using primers Apu35s2F 5′-TCTCAGATCG-GACCATCACATCAAT-3′ and Apu35sR 5′-TG CAGGTCCGATCTGAGACTTTTCA-3′, which generated a 300 bp fragment.

ELISA

The *npt* II product (NPTII) was detected using the Complete Kit PSP 73000/0288 (Agdia, Elkhart, IN, USA). Leaf clusters were prepared for every line represented by collecting two leaves from each replicate of the plant; leaves were ground and processed following the manufacturer's instructions. Positive and negative control tissues supplied in the kit were prepared by rehydration in the provided phosphate buffer just prior to use and processed following the manufacturer's instructions. Optical densities values (OD_{650}) were determined in a Multiskan EX (Thermo Scientific, Waltham, MA, USA) and data from negative samples were subtracted from sample data.

Chitinase activity

Chitinase activity was determined in the most tolerant transgenic plants using total proteins extracted from 100 mg of fresh young leaf tissue from the field, ground in 1 mL of extraction buffer (100 mM acetate buffer; pH 5.0, 0.5 M NaCl, 1 % (w/v) polyvinylpyrrolidone (PVP40), 0.1 % Triton X-100, 2 % 2-mercaptoethanol, and 20 % glycerol). The samples were incubated at room temperature for 3 h and then the supernatant was recovered by centrifugation at 5,000×*g* for 5 min. The total protein content was quantified using Coomasie Plus Protein Assay

Reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions. Chitinase activity (EC 3.2.1.14) was determined for each protein extract according to the procedure of Dana et al. (2006), with modifications. Assay mixes of 100 µL containing 1 µg of total protein extract and 250 µM of the fluorescent substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4-MU GlcNAc, Sigma-Aldrich Co, St. Louis, MO, USA) in 100 mM sodium citrate buffer, pH 3.0 were incubated for 15 min at 30 °C in the dark. Next, 2.9 mL of 0.5 M Gly-NaOH buffer, pH 10.4 was added to stop the reactions and fluorescence was measured at 350 nm excitation and 440 nm emission wavelengths in a Shimadzu RF-5301 PC fluorimeter (Shimadzu Corporation, Kyoto, Japan). Three measurements were performed for each transgenic plant using three independent protein extracts and chitinase activity was expressed in relative fluorescence units.

Qualitative assays of berry extracts

Selected lines from the top-ranked list were chosen and all of their clusters collected from their corresponding sources in the MB (first experiment). Upon reaching 18° Brix fruits were cut, ground and the berry juice was collected. One hundred µL of juice extracts were added to previously dispensed PDA Petri dishes and inoculated using 10^5 spores of *B. cinerea*. Dishes were incubated at 20 °C under a 16/8 h light/darkness photoperiod for 10 days and fungal growth visually evaluated.

Results

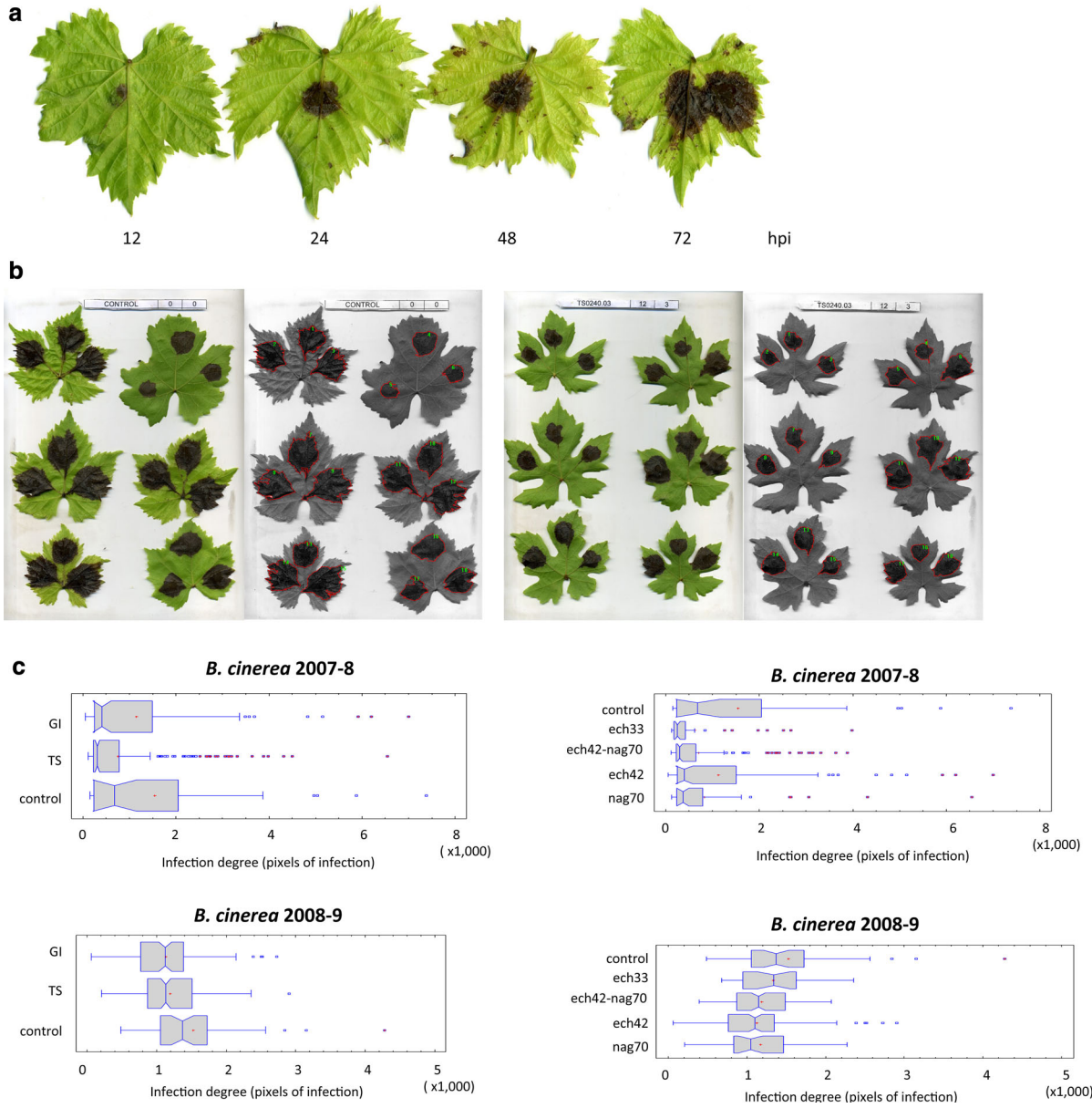
Tolerance to *B. cinerea*

A pattern of *B. cinerea* infections on grape leaves was first defined by application of agar slices from fully grown *B. cinerea* Petri dishes on leaves of non-GM TS field plants (n = 60 in the MB). Since necrotic spots of approximately 2 cm in diameter were obtained after 72 h (Fig. 1a), this time was defined as an adequate duration to generate noticeable infections in control plants and was considered suitable for comparison with the symptoms produced in GM plants from the MB (Fig. 1b). Analysis of the necrotic areas caused by *B. cinerea* challenges of the 103 GM TS lines (Fig. 1b; 568 plants divided into TS and GI groups) revealed that (at *P* value <0.05) both TS and GI groups had smaller lesions than the control plants in 2007–2008 and 2008–2009 (Fig. 1c). Although differences in the response of GM plants were observed in 2007–2008 by explant source (TS or GI), these differences were reduced in the second season. Using the same analysis, GM individuals for *ech*42 and *nag*70 genes and their combinations resulted in more tolerant individuals than the control individuals in both seasons. Interestingly, the GM individuals for the *ech*33 gene were tolerant during the first evaluation season but their tolerance was less pronounced in 2008–2009 (Fig. 1c).

Chronological improvement in the precision of the infection assays was seen over the time (see the results distribution in Fig. 1c) and most of the outlier GM individuals in 2007–2008 were included into their contiguous quartiles (mostly Q4) of their corresponding populations. Individuals that consistently occupied Q1 or were present in the lower whisker, i.e., the lowest 25 % of the data for each group throughout the trial, were filtered by explant group and ranked from most tolerant to least tolerant TS or GI plants, allowing for the establishment of a *B. cinerea* tolerant selection within each GM group.

Tolerance to *E. necator*

Screens for the natural presence of *E. necator* (Fig. 2a) in the MB was conducted. Visual inspections were carried out during the early stages of the 2007–2009 seasons for whitish or grayish patches on leaves (Fig. 2b; panels 1 and 3). Later in the season, the occurrence of powdery black spots and finally full leaf coverage were observed (Fig. 2b; panels 5, 7, and 9). All of the control plants exhibited an invariable infection rate of 9 late in the first season (2007–2008), showing unlimited patches or totally attacked leaf blades, strong sporulation, and abundant mycelium (Fig. 2c). In the same period, the transgenic populations showed a different response (*P* value <0.05) and ranked mostly at the 7 level (91 % GI and 93 % TS; Fig. 2c), exhibiting basically the same symptomatology as the controls, with the exception of zones with vast limited patches. During this same analysis, no differences associated between transgenes were detected and the mean values were 7. Interesting outlier individuals transformed with the double



(*ech*42 + *nag*70) construct exhibited low infection rates (3) (Fig. 2c).

In the second season, the control plants again exhibited an invariable very high disease infection rate (Fig. 2c), whereas the transgenic plants had a lower infection rate (P value <0.05). GM plants ranked between 5 (limited patches; 39 % GI and 40 % TS) and 7 (plants with vast patches, some limited, with strong sporulation, and abundant mycelium; 45 % GI and 47 % TS) (Fig. 2c). Some GM individuals reached maximum disease levels (i.e., 9; 6 % GI and 8 % TS) and a few lines had an infection rate of 5 or lower (10 % GI and 5 % TS). In terms of transgenes, whereas GM individuals obtained with the *ech*33 gene had a median value of 5 (i.e., patches usually limited to a diameter of 2–5 cm), GM individuals generated with *ech*42 or *nag*70 genes and their combination showed median values of 7 (i.e., vast patches, some limited,

◀**Fig. 1** *Botrytis cinerea* infection on leaves from GM TS plants. Leaves from field-grown plants were collected and inoculated using *B. cinerea* agar slices obtained from full growth in Petri dishes. Time course analyses of *B. cinerea* infections were first conducted in order to define the best inoculation time using wild-type plants ($n = 60$) and data acquisition was set according to the infection pattern in these plants in which spots reached 2 cm in diameter, usually after 72 h post-infection (hpi) (**a**). Infections were measured as necrotic spots (in pixels) by image scanning of challenged leaves (**b**); responses in control plants are shown at left. Six to eight leaves per plant were challenged (**b**) and each plant was evaluated at least in triplicate each season; images were acquired (**b**, *color pictures*) and processed for automatized necrotic area quantification (**b**, necrotic areas with *red borders* in *black-white images*). *Box-and-whisker plots* for infection pixels (**c**) during two seasons (2007–2008 and 2008–2009) of evaluations per explant source (*left plots*) and gene used (*right plots*) were derived from ranked data sets processed using the Kruskal–Wallis test in which medians in the columns (*stripes*) were compared. The average rank for these values (*red symbols*) was computed to identify medians that were significantly different from each other. Statistically significant differences among the medians at the 95.0 % confidence level were defined when the *P* value was <0.05. GI and TS are GM TS plants from somatic embryos obtained from two different sources, as indicated in the "Materials and methods" section

strong sporulation, and abundant mycelium) (Fig. 2c). No outliers were observed during these analyses and individuals with a minimal score of 3, displaying low infection with very limited patches, sporulation and mycelium, were scored for all of the exogenes used in the experimentation (Fig. 2c).

These analyses revealed the possible occurrence of combined tolerant lines to *B. cinerea* and powdery mildew. Plants with either *ech*42 or *nag*70 genes frequently showed differential tolerance to single infection by *B. cinerea* or powdery mildew, while plants with *ech*42 + *nag*70 and *ech*33 genes were the most tolerant to infection with both fungi.

Second field trial of GM 'Thompson Seedless'

A preliminary selection of the best 20 GM individuals sharing both tolerances was done (Table 1) and used in the design of a new field that mimicked commercial settings (Fig. 3a). The resulting field was successfully established in spring 2009, with 19 of these 20 selected lines (Fig. 3a–d) in which most of them contained *ech*42 gene. Five replicates from each original plant were randomly planted and this population was further characterized. Interesting phenotypic differences in some of the selected lines (23 and 35) were observed, in which plant size (Fig. 3c) and cluster shape (Fig. 3d) were markedly affected.

The transgenic status of the 19 lines with the highest tolerance to fungal infection was confirmed in leaf tissue by ELISA for NPTII (Fig. 3d) and NPTII immunostrips (data not shown) carried out in 2011. When these top-ranked individuals were assayed for total chitinase activity in 2011, non-homogeneous behavior was observed (Fig. 3e); whereas line 35 showed similar total activities compared with control plants, other lines (14, 23, 25, 33, 71, 74, 101, 114, and 115) showed total chitinase activities above those of the control individuals. Molecular analyses to corroborate transgene insertions were carried out in 2012 (Southern blot; SB; Fig. 4a) and transgene CN estimation in 2013 (qPCR; Fig. 4b). The presence of transgenic traits could not be confirmed in six out of 19 lines by any of these procedures (lines 8, 19, 23, 25, 114, 115; Table 1), including the double *npt*II—antifungal exogene assays in the case of qPCR. In addition, two lines obtained with *ech*42 (5 and 33) were confirmed by the qPCR detections but not by hybridization. Together, 11 out of 19 lines were confirmed for transgene insertion using both techniques; seven for *ech*42 (lines 14, 18, 20, 21, 49, 71, 84, and 160), two for the double *ech*42-*nag*70 (35 and 74), one in the case of *ech*33 (71), and one using the *nag*70 (101) gene construct (Table 1). SB detections of genomic DNA/*Hind*III digestions hybridized with a *nptII* probe showed that this latter group presented between 1 (line 14) and at least 4 insertions (line 18) of this gene, with a size range between 1 and 16 kb (Fig. 4a). On the other hand, determinations using qPCR of *ech*42, *nag*70, *ech*33, and *nptII* revealed the occurrence of six lines with CNs between 1 and 2 (lines 5, 14, 33, 35, 71, and 101; Fig. 4b) and seven lines with more than 2 transgene copies (lines 18, 20, 21, 49, 74, 84, and 160). The calculated CN of qPCR detections for different transgenes integrated within each line was not significantly different (Fig. 4b), with the only exception for *npt*II and *nag*70 in line 35. Furthermore, SB analysis revealed that several plants presented hybridization bands with smaller sizes than expected for *Hind*III digestions when complete T-DNA insertions were assumed (i.e., hybridization bands under 2.6 kb for *ech*42, *nag*70, and *ech*42-*nag*70 and bands under 3.2 for *ech*33 events). In addition, *npt*II qPCR data of GM lines for *ech*42

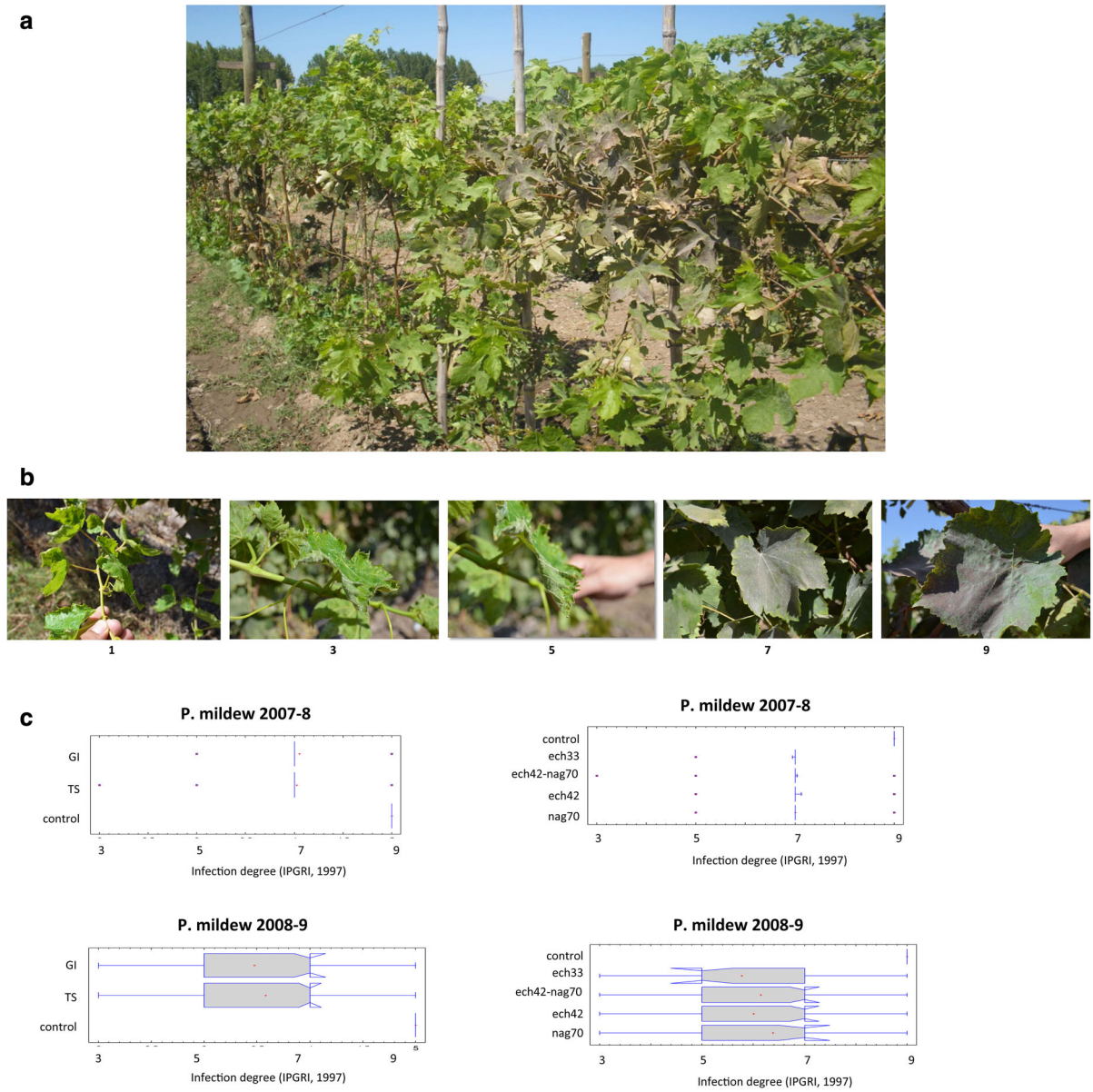


Fig. 2 Reaction of transgenic 'Thompson Seedless' to infection by *E. necator* in the field. All plants in the field (**a**) were inspected twice in 2007–2008 and 2008–2009 and scored for disease symptoms (**b**) such as *whitish and grayish patches* on leaves at the early stages and *black spots* later in the season using the IPGRI modified scale (UPOV, OIV 1997) with values ranging from very low (*1*) to very high (*9*) infection rates. *Box-and-whisker plots* for infection degrees (**c**) during two seasons (2007–2008 and 2008–2009) of evaluations per explant source (*left plots*) and gene used (*right plots*) were derived from ranked data sets processed using the Kruskal–Wallis test in which medians in the columns (*stripes*) were compared. The average rank for these values (*red symbols*) was computed to identify medians that were significantly different from each other. Statistically significant differences among the medians at the 95.0 % confidence level were defined when the *P* value was <0.05. GI and TS are GM TS plants from somatic embryos obtained from two different sources, as indicated in the "Materials and methods" section. All of the plants in the field were scored twice each season

construct cannot be considered suitable, due to technical problems affecting primer/probe attachment on the *nptII* sequence of that construct (represented by dashed lines in Fig. 4b). It is worth stressing that the consistent CN estimates of *npt*II and the other transgenes in qPCR (Fig. 4b) would indicate that the

Table 1 Most tolerant transgenic lines successfully established for additional molecular characterization

Line no.	Gene	Transgenic status confirmed by
5	Chi42	qPCR
8	Chi42-cNAG	None
14	Chi42	qPCR–SB
18	Chi42	qPCR–SB
19	Chi42	None
20	Chi42	qPCR–SB
21	Chi42	qPCR–SB
23	cNAG	None
25	cNAG	None
33	Chi42	qPCR
35	Chi42-cNAG	qPCR–SB
49	Chi42	qPCR–SB
71	Chi33	qPCR–SB
74	Chi42-cNAG	qPCR–SB
84	Chi42	qPCR–SB
101	cNAG	qPCR–SB
114	Chi42-cNAG	None
115	Chi42-cNAG	None
160	Chi42	qPCR–SB

qPCR real-time PCR, *SB* Southern blot, *None* neither SB nor qPCR allowed for successful GM confirmation

full-length T-DNA was consistently integrated in these lines.

These results show the generation of *B. cinerea* tolerant transgenic plants bearing chitinase activity over the controls (lines 14 and 101) with one or two copies of *ech*42 and *nag*70, respectively. In addition, lines with chitinase activity comparable to the controls (lines 21 and 160) with more than two copies of *ech*42 have been also generated. Antifugal activity of fruit juices from these specific lines was assayed by supplementing Petri dishes with *B. cinerea*-containing PDA with corresponding fruit juices. Clusters from transgenic and control vines were harvested in the MB and 100 µL of juice extracts were used for the assay to determine *B. cinerea* growth after 10 days of incubation. Differences in fungal growth were observed between treatments with fruit juice from transgenic clusters compared to the controls (Fig. 5a). The effect of juice from transgenic berries on fungal growth was confirmed by microscopy observations and showed a substantial decrease in the reproductive structures of the fungal mycelium (Fig. 5b). Interestingly, all four media supplemented with each of the different selected GM fruit extracts led to a decrease in these light-dense structures, producing the clear areas observed in the growth dishes (Fig. 5a).

Discussion

In this study, GM grapevines tolerant to *B. cinerea* and *E. necator* were identified by a first screening of 568 individuals followed by a further characterization of individuals exhibiting the highest tolerance level. This stepwise process lasted more than 8 years and relied on statistical analyses of plant reaction to fungal infections in the field and detached leaf assays for the grouping of individuals based on tolerance to both *B. cinerea* and *E. necator*. Although only preliminary data about GM status were initially linked to tolerance, the data obtained after three seasons of evaluations produced consistent information about the gradient of tolerance. The transgenic status of these plants was later confirmed using commercial antibodies for the detection of the *npt*II gene product, chitinase activity assays in the leaves and qPCR amplifications for transgene CN evaluation. This characterization generated evidence for the insertion and the expression of the transgenes (*ech*33 or e*ch42* and/or *nag*70) and marker gene (*nptII*) in the majority of the selected lines and consistently supported the phenotypic results deduced from the statistical process.

Suitability of transgenesis for fruit crop improvement largely relies on the production of individuals possessing satisfactory agronomic traits. Along with effects caused by tissue handling, regeneration and clonal propagation, these new crops need attention on both the possible pleiotropic effects of integrated DNA and the influence of the integration site and transgene architecture. Whereas these latter effects can be documented by molecular characterization studies such as CN and exogene expression, long term field studies allow for the identification of stability issues and eventual pleiotropic effects (Bolar et al. 2000; Pons et al. 2012). For instance, little seasonal variation was observed in the expression levels of markers genes, i.e., β-glucuronidase (*uid*A) and *npt*II, in citrus trees among plants of the same transgenic line in different organs and over 3 years (Pons et al. 2012). In addition, in different studies on transgenic trees, transgenic traits were less stable than expected

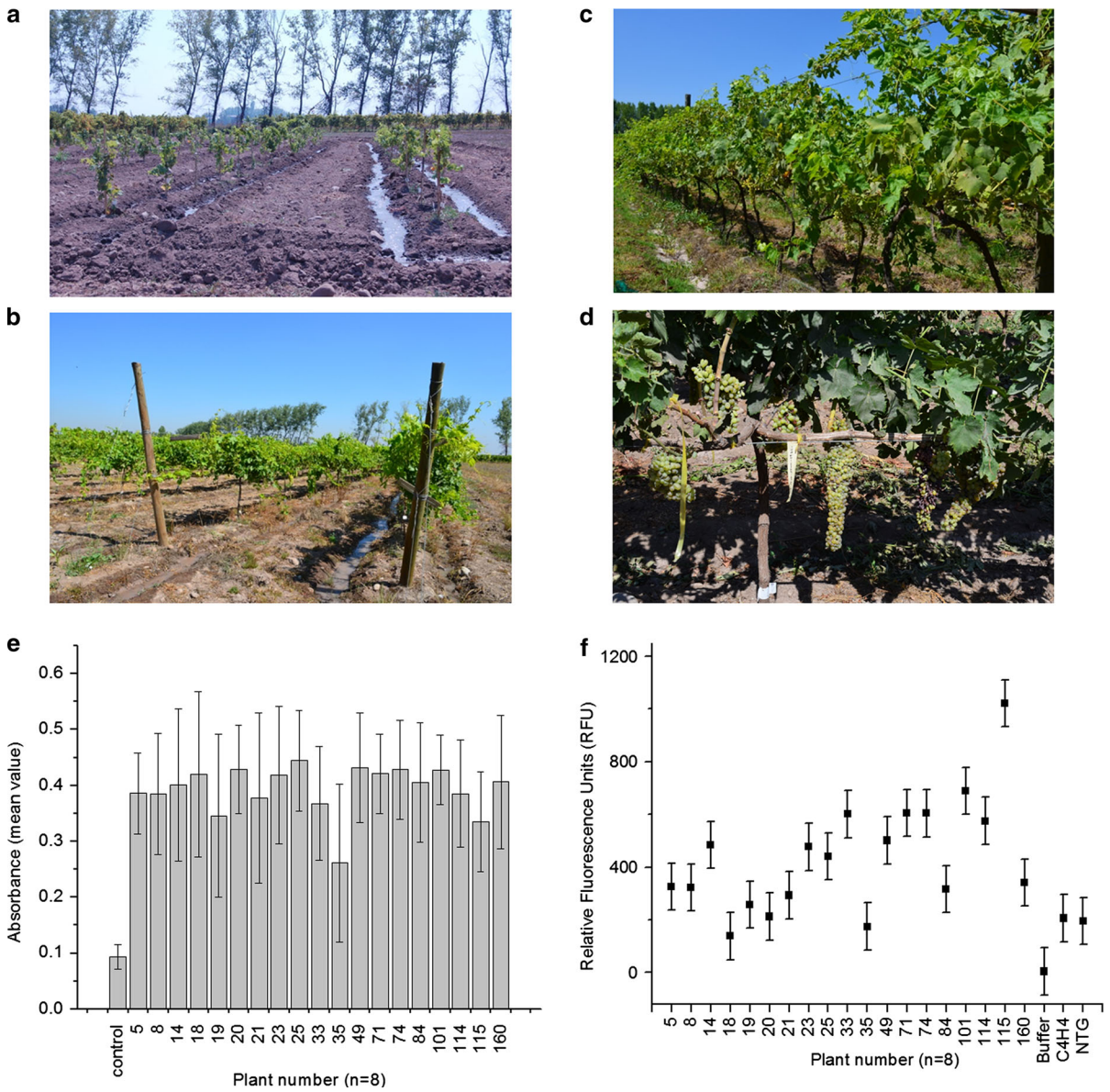


Fig. 3 Characterization of transgenic 'Thompson Seedless' plants that are tolerant to *B. cinerea* and powdery mildew. Individuals exhibiting the highest tolerance to both fungi were planted in a field mimicking commercial settings in 2009–2010 for further molecular characterization. Five plants representing each selected tolerant line were grafted onto Harmony rootstocks and planted to form a random block with control non-GM 'Thompson Seedless' (TS) plants (**a**). Field status for the 2012–2013 season (**b**), phenotype differences in plant size (**c**) and clusters shapes (**d**) were also obtained in some specific lines (dwarf line 35 is shown). Absorbance from ELISA detections of the neomycin phosphotransferase II protein (**e**) and chitinase activity assays (**f**; means for relative fluorescence units by plant number are shown with 95 % LSD intervals) over all of the selected individuals were evaluated in leaf batches from each plant group representing the corresponding GM line

(Höenicka and Fladung 2006; Flachowsky et al. 2008). In the present study, differences in plant size (Fig. 3c) and changes in cluster morphology (Fig. 3d) have been seen in some of the selected transgenic individuals after 3 years of observation for the resistance phenotype (second experiment). These results demonstrate the importance of detailed agronomic-oriented studies for properly screening interesting

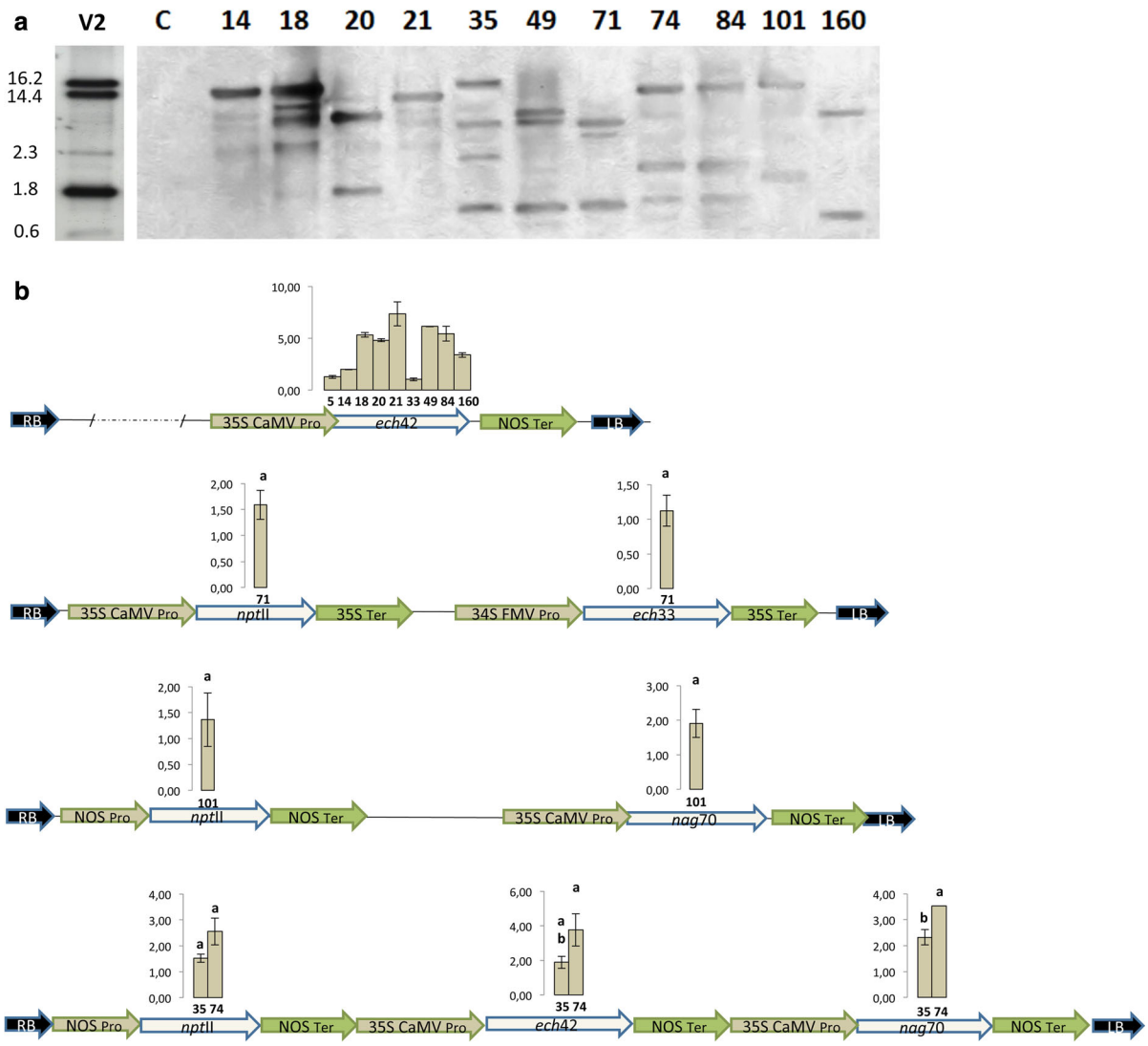


Fig. 4 Transgene copy number detections in the selected 'Thompson Seedless' population using Southern blot and qPCR techniques. Selected tolerant lines (Table 1) were analyzed for *npt*II transgene insertion by Southern blot (SB) hybridizations (**a**) confirming the transgenic status for 11 of the 19 selected lines (*numbers* indicate the corresponding line, *C* DNA from a non-GM plant, *V2* molecular size marker). Alternatively, genomic DNAs were subjected to copy number (CN) determination of each transgene (**b**) based on qPCR; plots on exogene cassettes for each construct (from *top* to *bottom ech*42; *ech*33; *nag*70; *ech*42-*nag*70) are shown indicating the CN (*y*-axis) per line (*x*-axis). Similarly, determinations of the *npt*II cassette (when possible) are included. The data were subjected to multiple comparisons using Tukey's honest significant difference test ($\alpha = 0.05$)

phenotypic variability, as also shown by Pons et al. (2012).

Similarly, the ELISA and *npt*II immunostrip results obtained during 2011–2012 season (second experiment with selected lines) confirmed data from the preliminary PCR screening done on in vitro plants leaves carried out during kanamycin selection (a pre-MB step); however, a more thorough molecular characterization of the 19 selected lines confirmed the transgenic status for 11 of these lines in 2012 and 2013 analyses using both SB and qPCR assay. Such discrepancies could be explained by the likely chimeric nature of these regenerated transgenic plants, an outcome previously reported (Dalla Costa et al. 2009).

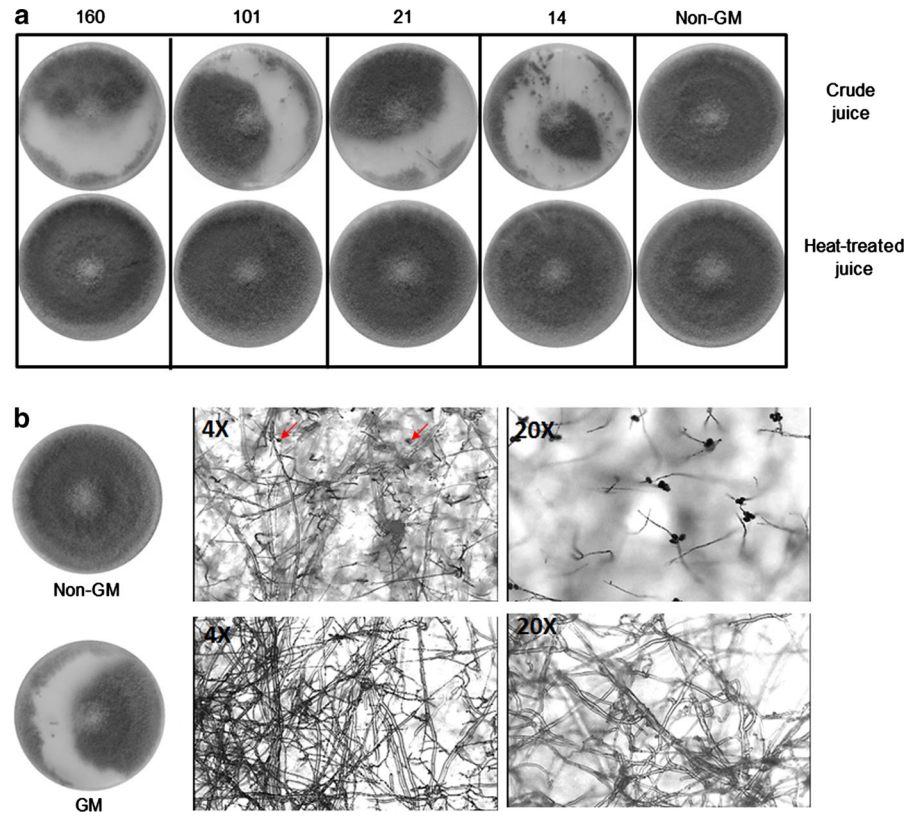


Fig. 5 Antifungal activity assays on GM lines showing the highest tolerance to *B. cinerea*. The antifungal activity of grapes was determined by comparing *B. cinerea* growth 10 days after incubation of fruit juice extracts from GM and non-GM plants or heat-inactivated GM fruit juices on fungal mycelium-containing PDA Petri dishes (**a**). PDA slides without light-dense areas from Petri dishes containing fruit juice from GM plant were analyzed by microscopy observations (**b**); limited or no reproductive structures were found (red arrows in controls). The relevant magnifications are indicated in the *upper left of each panel*

Different studies have reported that transgenic traits may get lost during subsequent generations (Bhat and Srinivasan 2002; Vain et al. 2002; Butaye et al. 2004), as well as during the cycles of vegetative propagation, particularly occurring in fruit trees (Flachowsky et al. 2008). In addition, successful examples of new varieties generated by gene transfer techniques in fruit crops such as plum (Hily et al. 2005) and papaya (Ming et al. 2008) have revealed that T-DNA insertions into host genomes resulted in more complex arrangements and insertions than expected. Values of transgene CNs within the tissue of the same line obtained in this work may suggest the presence of chimeric insertion. As previously pointed out (Dalla Costa et al. 2009), the combination of SB and qPCR enable the discovery of such outcomes and, according to CN values, suggest the presence of cell mixtures where GM and not GM cells may coexist in the tissues of the same plant. These results could be the drawback of somatic embryo gene transfer since multicellular origin of somatic embryos has been proposed for grapes (Quiroz-Figueroa et al. 2006; Martinelli and Gribaudo, 2009) and other fruit crops (Faize et al. 2010; Flachowsky et al. 2008).

Moreover, exogene chimeras and escapes may be the results of transient expression of the selectable marker gene (in this case *nptII*) during the early stages of the regeneration process (pre-MB step), the presence of *R. radiobacter* cells in the infected tissues, and/or the protection of non-transgenic cells by the surrounding GM cells (Domínguez et al. 2004). In the present work, 16 mg/L of kanamycin was used for primary selection of GM lines, as suggested by Dutt et al. (2007). This dosage proved not stringent enough, since individuals that were "non-homogeneous" for the transgenes were obtained. With these considerations taken into account, all of the tolerant lines defined in the present work have been re-introduced into further somatic embryogenesis analysis with the goal of avoiding the regeneration of chimeric plants by permanent selection pressure.

A marked predominance of *ech*42 transgenic individuals was found within the most tolerant population (Table 1). In comparison, the *ech*33 cloned in this

work from a local *T. virens* teleomorph isolate was represented by just a single transgenic line. Regardless of the transgene insertion and position effects, there are at least two causes that might lead to these results: a) different species of *Trichoderma* and isolates of same species have been described to differ in their biocontrol potential and chitinase activity (Sharma et al. 2012), suggesting more active candidate genes, and/or b) differential gene expression levels caused by promoters (35*S* vs. 34*S*) that behave differentially in *Vitis* spp. In a simplistic rationale, this latter alternative can be excluded by the fact that the other two 35*S* driven constructs (i.e., *nag*70 and *ech*42-*nag*70) were scarcely represented; in addition, this issue must be specifically addressed by further qPCR evaluations. On the other hand, since the pioneering work of Lorito et al. (1998), *ech*42 has been shown to be by far one of the most functionally active *Trichoderma* spp. genes in fruit trees conferring disease tolerance/resistance phenotypes. Expression of *ech*42 gene increased resistance to *V. inaequalis* with reduced plant growth in apples (Bolar et al. 2000) and in lemons led to enhanced resistance to *Phoma tracheiphila* and *B. cinerea* with a significant correlation between resistance and transgene expression (Gentile et al. 2007; Distefano et al. 2008). The presence of predominant *ech*42 individuals among the most tolerant lines may be consistent with works where the use of this gene led to successful generation of fungal tolerant fruit crops.

The convergence of genome sequencing on *V. vinifera* ('Pinot Noir' (Jaillon et al. 2007; Velasco et al. 2007) and TS (Di Genova et al. 2014)) with improved somatic embryogenesis for high throughput gene transfer methods (Tapia et al. 2009) will result in an enhanced evaluation opportunity for both new candidate genes and regulatory sequences from *Vitis* spp. (Gray et al. 2014). Recently, a *V. vinifera* thaumatin-like protein gene (*vvtl*-1) was cloned from 'Chardonnay' and used in the generation of 'TS' lines that showed delayed powdery mildew infection in greenhouse screenings and reduced severity of black rot disease in field tests (Dhekney et al. 2011). In addition, this approach is expected to avoid those secondary effects and reinforce the impact of gene transfer methodologies in the improvement of *Vitis* spp.

Botrytis cinerea is a major post-harvest problem in grapes, timely maturity of transgenic lines has just allowed for the evaluation of fruits. Berry juices from the most tolerant plants demonstrated an important inhibitory effect in *B. cinerea* growth assays. These results support the phenotyping and genotyping pipeline described in the generation of the selected population, which, in addition, agrees with the observations obtained by light microscopy.

In conclusion, after 8 years of field establishment, we have successfully identified a GM population of grapevines that shows enhanced tolerance to both *B. cinerea* and *E. necator*. The experimental data generated support these results, however to further characterize the agronomical behavior of the selected individuals is necessary, as described for transgenic citrus trees (Pons et al. 2012). Due to the possibility of transgene chimerism among the lines, additional somatic embryogenesis combined with a stringent transgene selection and evaluation may be needed in order to obtain new individuals with a more stable (and possibly more effective) presence of these tolerance traits.

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